Genetic modification of an entomopoxvirus: deletion of the spheroidin gene does not affect virus replication in vitro

Christopher P. Palmer,† Davin P. Miller, Susan A. Marlow,‡ Louise E. Wilson, Alison M. Lawrie and Linda A. King*

School of Biological and Molecular Sciences, Oxford Brookes University, Gipsy Lane Campus, Oxford OX3 0BP, UK

In the late stages of an entomopoxvirus infection, virions become embedded within a crystalline occlusion body or spheroid. Spheroids are composed primarily of a single polypeptide, spheroidin. We describe the construction of a genetically modified *Amsacta moorei* entomopoxvirus (AmEPV) in which the spheroidin gene coding sequences are deleted and replaced with those of a heterologous reporter gene encoding chloramphenicol acetyltransferase (CAT). A transfer vector, pAmCP1, was prepared containing a unique *BamHI* site in lieu of the spheroidin gene coding region, together with 1 kbp of upstream and downstream DNA sequence that flanks the spheroidin gene. The flanking sequences provide the transcriptional control signals and also guide homologous recombination so that the spheroidin gene coding region can be replaced with that of the foreign gene. The transfer vector was designed so that the translational start codon of the introduced foreign gene would be utilized. A recombinant virus, AmEPV.CAT, was produced by transfecting AmEPV-infected cells with the transfer vector encoding the CAT gene. The recombinant virus was isolated from wild-type virus by identifying plaques with a spheroidin-negative phenotype. Light microscopy and SDS-PAGE analysis demonstrated that no spheroids or spheroidin protein were produced in the recombinant virus-infected cells. The recombinant virus was able to replicate to high titres (10⁷ p.f.u./ml) in insect cells indicating that the spheroidin gene is non-essential for AmEPV replication in vitro. Moderate levels of CAT were synthesized in recombinant virus-infected cells and temporal analyses indicated that CAT synthesis followed the pattern of spheroidin production suggesting that the spheroidin gene promoter was functioning under normal regulatory control in the genetically modified virus.

**Introduction**

Entomopoxviruses (EPVs) are a group of insect-specific DNA viruses that have been classified as members of the *Poxviridae* family (Francki et al., 1991). The EPVs infect a number of insects that are regarded as pests in the orders Lepidoptera, Coleoptera, Orthoptera and Diptera, and their use as biological control agents is under investigation (Woods et al., 1992). The type member of the viruses specific for Lepidopteran insects is *Amsacta moorei* (Am) EPV, and largely because of the ability of this virus to replicate in vitro, it has been the most widely studied. AmEPV has a large dsDNA genome of about 225 kbp that is unusually A + T rich (81.5 %) (Langridge et al., 1977; Hall & Hink, 1990). Studies on the replication of AmEPV in cell lines derived from *Estigmene acrea* (EAA.BTI cells; Granados, 1981; Langridge & Roberts, 1982; Marlow et al., 1993) and *Lymantria dispar* (LD652 cells; Goodwin et al., 1990) have shown that the virus has a biphasic replication cycle, producing both occluded and non-occluded virus particles in the cytoplasm of infected cells. By analogy with the insect baculoviruses (reviewed by Blissard & Rohrmann, 1990), it is presumed that the non-occluded virus is important for cell-to-cell transmission, and that the occluded virus is necessary for horizontal transmission between insects (Granados & Roberts, 1970). The occlusion body, or spheroid, of AmEPV has been shown to be composed primarily of a single major species of polypeptide, spheroidin (Bilimoria & Arif, 1979). The AmEPV spheroidin gene has been identified, cloned and sequenced (Hall & Moyer, 1991). These studies indicated...
that the spheroidin protein (114-8 kDa) is encoded by a 3-0 kbp open reading frame, preceded by a promoter region with a TAAATG motif typical of vertebrate poxvirus late gene promoters (Rosel et al., 1986; Patel & Pickup, 1987). Homologues of the AmEPV spheroidin gene have been identified in two other EPVs isolated from Choristoneura biennis and C. funiferana (Hall & Moyer, 1993). Spheroidin synthesis occurs late in the EPV replication cycle, forming a crystalline matrix into which the intracellular form of the virus particle (ICV) is embedded. Thus spheroidin appears to play a similar role to polyhedrin found in insect baculoviruses (reviewed by Blissard & Rohrmann, 1990). Earlier in the AmEPV infection cycle, virus particles are released from the plasma membrane, gaining an extra lipid membrane, to form the extracellular virus (ECV) (Granados, 1981; Marlow et al., 1993). These observations suggest that the formation of occlusion bodies may not be essential for virus replication in vitro. In this paper we report that replacement of the spheroidin gene coding region with that of the reporter gene chloramphenicol acetyltransferase (CAT) resulted in the production of a genetically modified virus that does not synthesize spheroidin or produce spheroid occlusion bodies. This virus replicates to high titres in LD652 cells and is able to express the CAT gene (cat) to moderate levels using the spheroidin gene promoter.

**Methods**

**Propagation of cells and virus.** E. aerea (EAA.BTI) cells (kindly provided by Dr F. W. Hink, Ohio State University, Columbus, Ohio, USA) were maintained in TC100 medium (J.R. Scientific or Gibco) supplemented with 10% fetal calf serum (FCS) and 1% lipid concentrate as supplied for insect cell culture (Gibco). L. dispar (LD652) cells (kindly provided by Professor R. Moyer and Dr R. Hall, University of Florida, Gainesville, FL, USA) were maintained in EXCELL 401 medium (J.R. Scientific) supplemented with 10% FCS. Both cell lines were maintained at 28 °C. AmEPV inoculum was routinely produced by the infection of the LD652 cells at a low m.o.i. (0.5 p.f.u./cell), and harvesting the culture medium (ECV) and cells (ICV) at about 10 days post-infection (p.i.). ICV was released from the infected cells by three rounds of freezing and thawing. The virus inoculum was stored at −80 °C and was titrated by plaque-assay in LD652 cells (Hall & Moyer, 1991). On occasion, AmEPV inoculum was produced by infection of EAA.BTI cells at a high m.o.i. (10 p.f.u./cell) and processed as described above. For experimental work, both the EAA.BTI and LD652 cells were infected at a high m.o.i. (10 p.f.u./cell). Virus DNA for the amplification reactions described below was isolated from purified AmEPV using field-inversion gel electrophoresis, followed by electroelution of purified DNA, using a modification (Palmer, 1993) of the method described by Hall & Moyer (1991).

**Construction of AmEPV transfer vectors.** Using the published sequence data for the AmEPV spheroidin gene (Hall & Moyer, 1991), polymerase chain reaction (PCR) primers (see Fig. 3a) were designed to amplify two 1 kbp DNA fragments that flank the AmEPV spheroidin gene coding sequence (see Fig. 2a). The upstream flanking sequence (primers 1 and 2, Fig. 3a) contained the putative spheroidin gene promoter and had HindIII (5') and BamHI (3') cohesive ends to facilitate insertion into the plasmid pT773 (Pharmacia), which had been previously digested with the same restriction endonucleases; this produced the intermediate plasmid pT773.SUR. In addition, primer 2 was designed to convert the spheroidin gene ATG to ATA, thus ensuring that the start codon provided by the foreign gene coding sequences would be utilized. Primers 3 and 4 (Fig. 3a) were used to amplify the downstream flanking region with BamHI (5') and EcoRI (3') cohesive ends. The amplified fragment was inserted into pT773.SUR, which had been previously digested with BamHI and EcoRI, to produce the AmEPV transfer vector pAmCP1 (Fig. 2a) with a unique BamHI site for the insertion of foreign gene coding sequences.

The PCR conditions for the amplification of both spheroidin gene flanking regions were as follows: 50 mm-KCl, 10 mm-NaCl, 01% Triton X-100, 0.25 mM-dNTPs, 0.25 µM each primer, 1.5 mM-MgCl₂, 2.5 units Taq polymerase (Promega), and 10 ng AmEPV DNA. The reaction mixtures were subjected to the following thermal cycles: 94 °C (5 min), 37 °C (2 min), 74 °C (3 min), one cycle; 94 °C (1.5 min), 37 °C (2 min), 74 °C (3 min), four cycles; 94 °C (1.5 min), 50 °C (2 min), 74 °C (3 min), 29 cycles; 94 °C (1.5 min), 50 °C (2 min), 74 °C (15 min), one cycle. Samples of the reaction mixtures were analysed by gel electrophoresis to confirm that amplification of the appropriate DNAs had taken place, and then the remainder of the sample was treated with proteinase K, phenol-chloroform extracted and ethanol precipitated (Innis et al., 1990). The sequence across the BamHI cloning site of pAmCP1 was analysed by DNA sequencing (Sanger et al., 1977) using a reverse oligo downstream of the BamHI site. This confirmed the integrity of the spheroidin gene promoter and the mutagenic conversion of the ATG to ATA (Fig. 3b). All DNA manipulations and Escherichia coli transformations were carried out using standard procedures (Sambrook et al., 1989).

The coding region of cat was isolated from pCM7 (Promega) by digestion with BamHI and was inserted into the BamHI cloning site of pAmCP1 to produce pAmDM.CAT (Fig. 2b). The orientation of cat with respect to the spheroidin gene promoter was confirmed by restriction enzyme mapping.

**Production and isolation of recombinant AmEPV.** LD652 cells (1 × 10⁶) were infected with AmEPV at an m.o.i. of 10 p.f.u./cell and after 1 h adsorption period were transfected with 1 µg pAmDM.CAT using lipofectin (Gibco). At 6 h post-transfection, the medium was replaced with fresh EXCELL 401/10% FCS growth medium. Six days post-transfection, the culture medium was harvested and titrated by plaque-assay in LD652 cell monolayers. Putative spheroid-negative plaques were identified using the aid of a light microscope, isolated and plaque-purified twice to produce the recombinant virus AmEPV.CAT. The integrity of the recombinant virus genome was confirmed by Southern blot analysis (Southern, 1977; Sambrook et al., 1989) using virus DNA isolated from purified AmEPV or AmEPV.CAT. The purified DNA was digested with ClaI or HindIII and analysed in a 0.8% agarose gel. The DNA was transferred to a nylon membrane (Hybond, Amersham) and hybridized using standard protocols (Sambrook et al., 1989), firstly to a radio labelled cat-specific probe (BamHI fragment from pCM7), and secondly to a radio labelled spheroidin-gene-specific probe (1.4 kbp PCR fragment of the 5' end of the spheroidin gene coding region; Palmer, 1993). Probes were labelled with [α-32P]dATP using the random oligonucleotide extension method.

**Analysis of cat expression in recombinant AmDM.CAT-infected cells.** LD652 cells (5 × 10⁶) were infected with AmEPV or AmEPV.CAT at an m.o.i. of 10 p.f.u./cell, or were mock-infected with medium, as previously described (Marlow et al., 1992). At various times p.i., the cells were harvested, washed in PBS and resuspended in 25 mm-Tris–HCl, pH 7.8. Following three cycles of freezing and thawing, the cell lysates were analysed for CAT activity using the standard [14C]chloramphenicol conversion assays (Gorman et al., 1982). Pilot
Genetic modification of AmEPV

Mol. mass (kDa)

(a)

NI

Am

S

116 -

96--

69-

46--

30-

21.5 -

14-3 -

(b)

(c)

Fig. 1. Analysis of spheroidin synthesis in wild-type and recombinant AmEPV-infected cells. (a) Coomassie blue-stained SDS-polyacrylamide gel showing the abundance of spheroidin protein (arrowed, S) in AmEPV-infected E. acrea (EAA.BTI) cells at 96 h p.i. E. acrea cells were infected at an m.o.i. of 10 p.f.u. AmEPV/cell (lane Am) or were mock-infected with culture medium (lane NI). Cells were harvested at 96 h p.i. and analysed by 10-20% gradient SDS-PAGE. Molecular mass markers are indicated in kDa, left. (b) Nomarski light micrograph showing the presence of spheroids (arrowed) in LD652 cells infected with wild-type AmEPV. (c) Nomarski light micrograph showing characteristic granulation of the cytoplasm but lack of spheroid production in recombinant AmEPV.CAT-infected LD652 cells. Bar, 20 μm.

Results

Deletion of the AmEPV spheroidin gene

Analysis of AmEPV-infected cells by SDS–PAGE shows that the major protein present in the latter stages of the infectious cycle is spheroidin (Fig. 1a), which forms the crystalline matrix of the spheroid occlusion bodies (Fig. 1b). The biphasic production of occluded and non-occluded AmEPV in LD652 and EAA.BTI cells suggest that the spheroidin gene may not be necessary for virus propagation in vitro. In order to investigate this possibility, we developed a system for the genetic modification of the AmEPV genome in which we are able to delete the spheroidin gene coding region.

The first stage in the production of a genetically modified AmEPV was the construction of an intermediate transfer vector, pAmCP1, which contained approximately 1 kbp of upstream (SUR) and downstream (SDR) AmEPV sequences that flank the spheroidin gene coding region (Fig. 2a). These flanking sequences provide the transcriptional promoter (SUR) and termination (SDR) signals, and also guide homologous recombination to replace the spheroidin gene coding sequences with those of a foreign gene. This is analogous to the system that has been developed for introducing foreign genes into the baculovirus genome at the polyhedrin gene locus (reviewed by King & Possee, 1992).

The AmEPV spheroidin gene has been sequenced and mapped to the HindIII ‘G’ fragment of the virus genome (Hall & Moyer, 1991). Using the data provided by this study, we designed four PCR primers (Fig. 3a) to amplify the 1 kbp SUR and SDR flanking regions of the spheroidin gene (Fig. 2a). As the spheroidin gene promoter has not been analysed in any detail, the 3’ primer of the SUR region was designed to incorporate 5’ non-coding sequences up to and including the AT of the initiation codon. The third nucleotide of the initiation codon was changed to A in the primer, so that translation of recombinant proteins would start at the ATG provided by the foreign gene coding sequence (Fig. 3b).
Fig. 2. Strategy used in the construction of the AmEPV transfer vector, pAmCP1. (a) The PCR primers used to amplify the upstream (primers 1 and 2, see Fig. 3a) and downstream (primers 3 and 4, see Fig. 3a) sequences flanking the spheroidin gene coding region are presented in relation to the HindIII map of the AmEPV genome. Primer 2 encodes a mutagenic change to convert the ATG sequence to ATA. The amplified upstream (SUR) fragment was inserted into pT7T3 to produce the intermediate plasmid pT7T3.SUR. The amplified downstream (SDR) fragment was then inserted into pT7T3.SUR to produce the transfer vector pAmCP1 containing the unique BamHI site for the introduction of foreign gene coding sequences. An arrow marks the direction of transcription. Hatched areas represent the PCR-amplified spheroidin upstream region (SUR) and spheroidin downstream region (SDR). (b) Restriction enzyme map of the transfer vector pAmDM.CAT showing the insertion of E. coli cat coding sequences from pCM7 (Promega; blackened region) into the BamHI site. The orientation of cat was confirmed by restriction enzyme mapping.

Following PCR amplification, the 1 kbp SUR was directionally cloned into the HindIII and BamHI sites of the plasmid pT7T3 to produce the intermediate plasmid pT7T3.SUR. The amplified 1 kbp SDR was then directionally cloned into the BamHI and EcoRI sites of pT7T3.SUR to produce the transfer vector pAmCP1 (Fig. 2a). This cloning strategy produced a unique BamHI site into which foreign genes could be inserted. The correct order of nucleotides upstream of the BamHI site, including the mutagenic change of ATG to ATA was confirmed by DNA sequencing (Fig. 3b).

Confirmation that the PCR-amplified 1 kbp SUR in pAmCP1 contained promoter activity was provided by inserting E. coli cat coding sequences into the BamHI site to produce pAmDM.CAT (Fig. 2b). When pAmDM.CAT was introduced into AmEPV-infected EAA.BTI cells, transient production of CAT was detected using an ELISA-based CAT detection system.
Genetic modification of AmEPV

(a) Primer 1
```
5' CCGCAAGCTTCACAGTACGCAACCTTTTGTGATCA'3'
```
HindIII
ATG to ATA

(b) Primer 2
```
5' GCGCGYGATCCGTTCCAAGGCTACGTTACTATTATG'3'
```
BamHI

Primer 3
```
5' CCGCGATCCCGCTAAATACGTTGGAGAATAAACAGG'3'
```
BamHI

Primer 4
```
5' CCGCGATTCCTCATAAGATTTACCACAAGATAG'3'
```
EcoRI

Fig. 3. (a) Sequence of the four PCR primers used in the amplification of the SUR (primers 1 and 2) and SDR (primers 3 and 4) DNA fragments. The introduced restriction enzyme sites near the primer ends are indicated and the mutagenic change of ATG to ATA is underlined in primer 2. (b) Results of the dideoxy sequencing across the BamHI cloning junction of pAmCP1. The transcription initiation triplet (AAA) is indicated, as is the mutagenic change ATG to ATA. Reverse complementarity is shown for reader convenience.

(data not shown). This result confirmed that the PCR-amplified SUR region contained spheroidin gene promoter activity.

Production of a recombinant AmEPV encoding cat

In order to make a recombinant AmEPV encoding Cat, LD652 cells were infected with AmEPV and then transfected with pAmDM.CAT as described in Methods. Recombinant virus should result from homologous recombination in the sequences flanking the spheroidin gene in the virus genome and cat in the transfer vector. As recombinant virus should no longer synthesize spheroidin protein, such viruses were isolated from wild-type by plaque-assay in LD652 cells and screening for plaques without spheroids. The frequency of putative recombinants viruses at this stage of screening was about 3%. As recombinants may be generated from single cross-over events to give false recombinants, as well as double cross-over events to give true recombinants (Mackett, 1992), each spheroidin-negative virus was amplified in LD652 cells and the crude DNA extracts were tested for the presence of cat by dot-blot procedures (data not shown). The overall frequency for the generation of true recombinant viruses varied between 0.1 and 0.2%, which is comparable to the frequencies originally obtained in the initial studies with the baculovirus (reviewed by King & Possec, 1992; O'Reilly et al., 1992) and vaccinia virus expression systems (reviewed by Mackett, 1992).

One recombinant, designated AmEPV.CAT, was amplified on a larger scale for further characterization. Light microscopy studies (Fig. 1 c) confirmed that no spheroids were formed in the recombinant virus-infected cells. In comparison, Fig. 1 (b) shows cells infected with wild-type AmEPV in which spheroids are clearly visible in the cytoplasm. The recombinant virus was able to replicate to titres (10^7 p.f.u./ml) similar to those obtained with wild-type AmEPV. The virus inoculum appears to be stable and has been successfully stored at -80 °C for over 1 year.

The integrity of cat within the recombinant virus genome was analysed by Southern blotting. DNA was extracted from purified AmEPV.CAT or wild-type AmEPV virions and digested with HindIII or ClaI. Southern blot hybridization indicated that cat hybridized to only the expected DNA bands of the recombinant virus genome (Fig. 4a). After removing the cat probe, the same blot was hybridized with a spheroidin gene coding region-specific probe. This highlighted only the expected restriction fragments of the wild-type AmEPV genome (Fig. 4b). These data confirm that in the recombinant virus genome, the spheroidin gene coding sequences have been replaced with those of cat.

Synthesis of CAT in AmEPV.CAT-infected cells

LD652 cells were infected with AmEPV.CAT, AmEPV or were mock-infected with culture medium. The cells were harvested at various times post-infection and
analysed for spheroïdin synthesis by SDS–PAGE (Fig. 5) or CAT activity by [14C]chloramphenicol conversion assays (Fig. 6). The results demonstrated the production of spheroïdin from 72 h p.i. in AmEPV-infected cells (Fig. 5); no spheroïdin was detected in the non-infected or AmEPV.CAT-infected cells (Fig. 5). No obvious band corresponding to the CAT protein was visible in the Coomassie blue stained PAGE gel analysing the AmEPV.CAT samples. However, several virus protein bands were detected in the region in which CAT would have been expected (about 27 kDa) and may, therefore, be masking the CAT band. Synthesis of CAT was,
products were separated by thin-layer chromatography. Autoradiograms were incubated with [l-14C]chloramphenicol and the acetylated products indicated. The cells were harvested and lysates prepared to measure the percentage of acetylated chloramphenicol. This was used to express CAT activity as nmol a4c converted to the acetylated products/min/mg cell protein (umol/min/mg). NI, non-infected cells; Am, wild-type AmEPV-infected cells at 96 h p.i.

therefore, analysed by activity assays as described in Methods. As expected, no CAT activity was detected in the non-infected or wild-type virus-infected cells (Fig. 6). In the AmEPV.CAT-infected cells, CAT activity was just detectable at 48 h p.i., attaining maximum levels at 96 h p.i. In a comparison with a recombinant baculovirus expressing cat, we found that the polyhedrin gene promoter (in AcRP18.CAT; gift of R. D. Possee, Oxford, UK) was about 10- to 20-fold more active than the spheroidin gene promoter in AmEPV.CAT (data not shown). Temporal regulation of CAT synthesis in the AmEPV.CAT-infected cells (Fig. 6) appeared to follow closely the pattern of synthesis of spheroidin in AmEPV-infected cells (Fig. 5), suggesting that the spheroidin gene promoter was functioning under normal regulatory controls in the recombinant virus-infected cells.

Discussion

The major protein synthesized late in the AmEPV replication cycle is the occlusion body protein, spheroidin, which forms the crystalline matrix into which virions become embedded. By analogy with the insect baculovirus polyhedra (Blissard & Rohrmann, 1990), spheroids are thought to be important for virus protection between insect hosts (Granados & Roberts, 1970), and ECV and ICV are thought to be important for cell-to-cell transmission both in vivo and in vitro (Granados & Roberts, 1970; Granados, 1981; Langridge & Roberts, 1982). In this study, we have developed a system for the genetic modification of the AmEPV genome at the spheroidin gene locus. This was achieved by constructing a transfer vector that would guide homologous recombination at the spheroidin gene locus within the virus genome, essentially replacing the spheroidin coding sequences with those of a BamHI restriction enzyme site. The BamHI site also facilitates the insertion of foreign gene coding sequences that can then be expressed under control of the spheroidin gene promoter. Previous studies by Hall & Moyer (1991) indicated that the spheroidin gene promoter contained a transcriptional start motif (ATAAATG) that is typical of those found in vertebrate poxvirus late gene promoters (Rosel et al., 1986; Patel & Pickup, 1987). The mRNA start site was mapped to the AAA region of this motif and transcripts were shown to have a 5' terminal poly(A) tail (Hall & Moyer, 1991). Although the spheroidin gene promoter has not been analysed in detail, we assumed that providing the complete sequence upstream from, and including, this motif would be sufficient for the spheroidin promoter to be active in a recombinant virus. By introducing a reporter gene (cat) into the BamHI site of the transfer vector, we were able to show in transient CAT assays that the amplified SUR contained promoter activity before attempting to make a recombinant virus.

As poxvirus DNA is non-infectious (reviewed by Moss, 1990; Mackett, 1992), a recombinant virus was produced by introducing the cat transfer vector (pAmDM.CAT) into AmEPV-infected cells. Initial studies demonstrated that the optimal time for transfection was at 1 h p.i. (Palmer, 1993). Putative recombinant virus was identified by its spheroidin-negative phenotype. However, as this phenotype may arise from single cross-overs, as well as double cross-overs (Mackett, 1992), the majority of the putative recombinant viruses did not contain cat. The recombination frequency for double cross-overs that integrated cat into the AmEPV genome was about 0.1-0.2%, which is comparable to the frequencies obtained in the initial stages of the development of both the vaccinia virus (Mackett, 1992) and baculovirus expression systems (King & Possee, 1992; O’Reilly et al., 1992). The integrity of cat, and the deletion of the spheroidin gene coding sequences, in the AmEPV.CAT genome was confirmed by Southern blot analysis (Fig. 4).

The recombinant AmEPV.CAT virus did not form spheroid occlusion bodies nor produce the spheroidin protein in virus-infected cells, but was able to replicate to titres similar to those obtained with wild-type virus, about 10^7 p.f.u./ml. From these data, we conclude that the spheroidin gene is non-essential for virus replication in vitro. This result is similar to that obtained during the development of the baculovirus expression system, when Smith et al. (1983a) demonstrated that deletion of the polyhedrin gene coding region did not affect the ability...
of baculoviruses to replicate in vitro. After the publication of these results, the polyhedrin gene locus became the target site for the introduction of foreign genes into the baculovirus genome (Smith et al., 1983b; reviewed by King & Possee, 1992; O’Reilly et al., 1992).

Examination of protein synthesis by SDS–PAGE demonstrated that the only major difference observed between the recombinant and wild-type virus, throughout the virus replication cycle, was the absence of spheroidin protein in the former (Fig. 5). All other proteins appeared to be similar in both the modified and unmodified virus. We were not able to conclusively identify CAT protein by SDS–PAGE, probably because of the appearance of several virus-specified proteins with a similar size to CAT (about 27 kDa). Pulse-labelling cells with [35S]methionine did not clarify the situation further (data not shown; Palmer, 1993). However, CAT activity assays demonstrated that moderate levels of CAT were being synthesized in the recombinant-virus infected cells. A time-course study indicated that the temporal regulation of CAT synthesis was similar to that observed for the synthesis of spheroidin, suggesting that in the recombinant virus the spheroidin gene promoter was under the same regulatory control as in the wild-type virus.

As shown in Fig. 1(a), spheroidin accounts for the majority of total cell protein in virus-infected cells at late times in the replicative cycle, with a yield that appears similar to that obtained with the baculovirus polyhedrin protein (e.g. see King & Possee, 1992). We had, therefore, expected that the amount of CAT activity detected in recombinant EPV-infected cells would approach that obtained in recombinant baculovirus-infected cells. Our experiments indicated, however, that much less CAT activity was obtained with the recombinant EPV. Although there may be several explanations for this, one reason may be that sequences downstream of the spheroidin transcription initiation site and, therefore, not present in the recombinant virus, may be important for optimal transcriptional activity. Alternatively, the mutagenic change of ATG to ATA may also have affected the levels of transcription from the spheroidin promoter. Evidence from studies on vaccinia virus late gene promoters have shown that changes to the AAA triplet severely diminish transcriptional levels, whereas mutations in the other bases of the consensus sequence, or in other sequences downstream, have limited effects on transcription (Davison & Moss, 1989). As the spheroidin gene promoter has not been characterized in detail, the importance of these sequences in determining insect poxvirus promoter activity remains to be determined. The results of the studies presented here, however, would indicate that the mutagenic change G to A and/or sequences within the 5’ end of the spheroidin gene coding region may be important for full promoter activity. This possibility is currently under further investigation.

We are also developing methods whereby the identification of recombinant viruses may be improved by using a blue-white selection method based on the E. coli lacZ gene or by making use of the luciferase gene (Richardson et al., 1992) (D. P. Miller & L. A. King, unpublished data). It may also be possible to enhance the recovery of recombinant viruses by utilizing AmEPV thymidine kinase (tk) mutants, as has been developed for the selection of vaccinia virus recombinants (Mackett et al., 1985). The AmEPV tk gene has been described (Gruidl et al., 1992), although a suitable tk− cell line would be required for this method to work.

While it is unlikely that an EPV expression vector system would ever gain the popularity of the insect baculovirus expression system, there may be specific instances where the insect poxvirus vectors may be useful. In particular, insect EPVs are currently being considered as biological control agents for a number of pest species (Woods et al., 1992), particularly species in those orders from which baculoviruses have never or only rarely been identified. As one of the main disadvantages of EPVs, like baculoviruses, is the long time taken to kill the host insect, the methodology that we have presented here for the genetic manipulation of the EPV genome provides a means for the incorporation of foreign genes that may improve the efficacy of these viruses as pest control agents.

We thank Professor R. Moyer and Dr R. Hall for the LD652 cells, Dr F. W. Hink for the EAA.BTI cells and Dr R. D. Possee for the virus AcRP18.CAT. This work was supported by grants from the UK SERC Biotechnology Directorate, AFRC and Zeneca Agrochemicals. C.P.P. and D.P.M. were supported by SERC studentships.

References


Genetic modification of AmEPV


(Received 25 June 1994; Accepted 8 September 1994)